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PRINCIPAL INVESTIGATOR: Timothy W. Synold, Ph.D.

CONTRACTING ORGANIZATION: Beckman Research Institute
Duarte, California 91010-3000

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Timothy W. Synold, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beckman Research Institute
Duarte, California 91010-3000

E-Mail: tsynold@coh.org

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Our experience studying the *MDR*1 gene prompted us to initiate work on a novel animal model to study *MDR*1/*mdr*1 gene expression under a variety of normal and breast cancer-related physiological conditions. With the advent of new bioimaging technology and the advancement of efficient gene targeting strategies, we found an opportunity to apply these state-of-the-art molecular tools to our problem. The work performed with the support of this grant has enabled us to; 1) engineere a targeting vector to allow insertion of a reporter (luciferase or HSV-tk) into the genomic locus of the mouse *mdr*1a gene; 2) create mouse embryonic stem cells in which a gene replacement/knock-in strategy was used to insert luciferase into the mouse *mdr*1a genomic locus; 3) demonstrate that luciferase expression in these cells requires Cre recombinase to bring luciferase in-frame with the translational start site of the *mdr*1a gene product; 4) show that the recombined configuration of *mdr*1/LUC, in its cDNA form, encodes a functional protein with luciferase activity, and 5) create both founder and Cre-recombinase expressing mouse strains for use in *in vivo* imaging experiments. Work performed to date has proved the feasibility of this approach. However, further refinements to the model are required.

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Table of Contents

Introduction

Transcriptional gene regulation represents the major mechanism of controlling gene expression and even subtle changes in expression can affect cellular functions in profound ways. Yet we have little direct knowledge of how genes are regulated in the whole body, due largely to an inability to observe and measure changes in gene expression under real physiological conditions and in real time. For similar reasons, the effects of given cancer therapies on their target(s), if mediated at the transcriptional level, have not been possible to study directly. However, the recent revolution in molecular imaging has led to the development of novel tools for performing noninvasive, *in vivo* imaging of gene expression. So far, this has been accomplished almost exclusively with a reporter gene linked to some length of promoter fragment and introduced either as a transgene or in the context of engineered cells injected into the animal. A more biologically relevant application of imaging technology would be to <u>target, through homologous recombination, a reporter gene into the genomic locus of a gene for the purpose of watching the regulated expression of that gene *in situ*, in real time, and in response to specific developmental, physiological, or environmental signals</u>. We proposed to establish such a system using the mouse *mdr*1a locus as a proof of principle and as a biologically important gene. Multidrug resistance (MDR) remains a serious impediment to curative chemotherapy in breast cancer patients. One mechanism of MDR is the enhanced expression of the human *MDR*1 gene product, Pgp.[1,2] *MDR*1 overexpression has been associated with drug resistance in many human cancers, but its contribution to clinical drug resistance remains unresolved.[2] Systematic longitudinal studies to determine *MDR*1's contribution to resistance are difficult, if not impossible, to perform in humans and an adequate animal model to study such questions has not been developed.

The main idea of this proposal was to use homologous recombination to place a bioimaging reporter gene such as luciferase (which can be detected using a bioluminescence instrument) or HSV-*tk* (detectable using state-of-the-art positron emission tomography probes and instrumentation) into the context of a genomic locus for purposes of studying gene expression *in vivo*, in real time, and in response to various drugs and developmental signals. The design of our targeting vector is such that locus-driven expression can occur only after Cre-mediated recombination brings the reporter in-frame with the translational start site of the gene being studied. Because Cre recombination can be controlled temporally and spatially, this strategy represents a novel approach to studying regulated gene expression specifically in breast tissues under defined conditions. We proposed using the mouse *mdr*1a chromosomal locus as the proof of principle, in part because there is a substantial literature describing its tissue-specific and inducible expression in a variety of contexts, thus providing an extensive base of knowledge against which to judge the validity of our model for studying regulated gene expression. At the same time, there are several important and controversial questions about *mdr*1 expression and breast cancer resistance that have, heretofore, been impossible to resolve using conventional molecular biology tools and a better model for studying this gene is needed.

Body

The work performed with the support of this grant has enabled us to: 1) engineer a targeting vector to allow insertion of a reporter (luciferase or HSV-tk) into the genomic locus of the mouse *mdr*1a gene; 2) create mouse embryonic stem cells in which a gene replacement/knock-in strategy was used to insert luciferase into the mouse *mdr*1a genomic locus; 3) demonstrate that luciferase expression in these cells requires Cre recombinase to bring luciferase in-frame with the translational start site of the *mdr*1a gene product; 4) show that the recombined configuration of *mdr*1/LUC, in its cDNA form, encodes a functional protein with luciferase activity, and 5) create both founder and Cre-recombinase expressing mouse strains for use in *in vivo* imaging experiments. Details of the work performed under each of the tasks outlined in the *Statement of Work* are provided below.
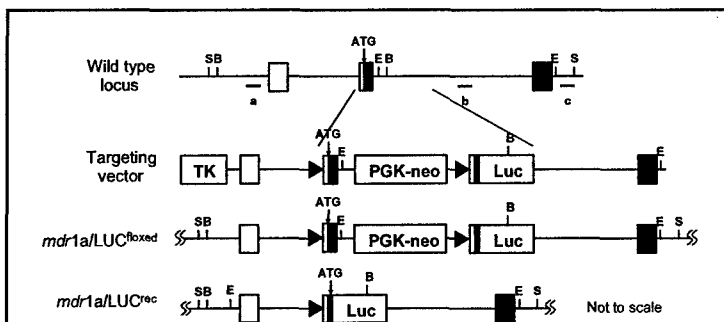
Statement of Work

4

Task 1;

   a. Obtain mdr1a genomic clone (exons 1-3 plus flanking sequences) from 129S1/svImJ mouse strain, compatible with the ES cells used in our Transgenic Mouse Facility.
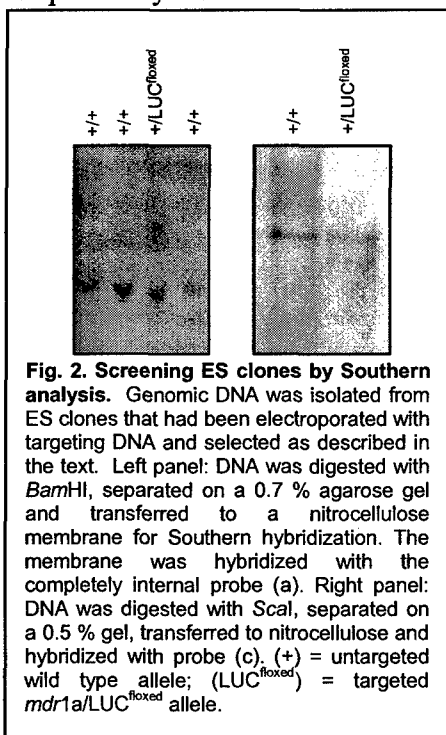
   b. Engineer PGK-neo and Renilla luciferase cassettes, already available, with appropriate loxP sites, into mdr1a locus. Repeat for HSV-tk reporter.

The targeting vector backbone (TG3; City of Hope Transgenic Mouse Facility) was designed to allow the insertion of two isogenic genomic DNA fragments ("arms") from the target gene. It contains an antibiotic expression cassette (Neomycin phosphotransferase) inserted between two loxP sites and the two arms of homology. This allows for positive selection of homologous recombinants and removal of the marker after Cre-mediated recombination. A negative selection cassette (Herpes Simplex Virus thymidine kinase) was included outside the 5' arm of homology to allow counter-selection against random integration of the gene-targeting vector.

The *mdr*1a/LUC targeting vector (Fig. 1) was constructed in several steps. PCR primers were used to amplify a 1481 bp 5' homologous arm that incorporated 900 bp of the *mdr*1a promoter and this was inserted into the 5'-most cloning site of the TG3 vector backbone. A 291bp fragment that incorporated the *mdr*1a Kozak region and the first coding exon (exon 2) was amplified by PCR and inserted between the two loxP sites, u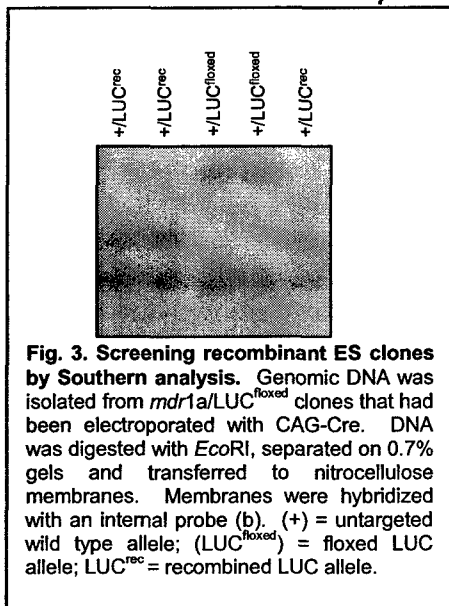pstream of the Neo cassette. A cloning strategy was then devised to fuse *Renilla* luciferase in-frame with the translated sequences of exon 2 of *mdr*1a and this fragment was inserted downstream of the loxP-neo-loxP cassette. Finally, the 3' homologous arm of *mdr*1a was amplified by PCR and this 6297bp fragment was inserted downstream of the LUC-exon2 cassette. The *mdr*1a/LUC allele in its unrecombined form is referred to as *mdr*1a/LUC^{floxed}. The *mdr*1a/LUC allele after Cre-mediated recombination is referred to as *mdr*1a/LUC$^{rec.}$

Fig. 1. Knock-in strategy. The map shows *mdr*1a exons 1-3, respective introns (thin line) and upstream genomic sequences. Exons in white are non-translated; exons in black are translated, with the initiating ATG indicated. Black bars (a-c) indicate positions of Southern hybridization probes. **Restriction sites:** B, *Bam*HI; E, *Eco*RI; S, *Sca*I. **PGK-neo:** *neo* under the control of the PGK promoter. **Luc:** *Renilla* luciferase fused in-frame with the translated sequences of exon 2 (black). **TK, thymidine kinase**, negative selection marker. **Arrowheads**: loxP sites, targets for Cre recombinase; the 5' lox site is engineered upstream of translation start (Kozak) sequences. The *mdr*1a/LUC^{rec} locus (bottom) is the gene configuration in cells expressing Cre recombinase. A single lox sequence remains upstream of the translation start site.

   *c. Give targeting vectors to Transgenic Mouse Facility for injection into ES cells, generation of mice, and initial screening for recombinants. **Deliverable** = mice with LUC and TK targeted to mdr1a locus, unrecombined.*

ES cells were generated at the City of Hope Transgenic Mouse Facility. The targeting vector was linearized, electroporated into ES cells and cultured for 9 days without selection. On the ninth day, individual colonies of clonal ES cells were selected, dissociated, and plated individually with STO feeder cells under neomycin selection for 4 days. ES clones were subsequently screened by Southern analysis for the homologous targeting event using internal and external probes (Fig. 2). From the 312 clones screened, one positive clone (132) was obtained. Clone 132 was expanded and re-screened to confirm the proper targeting

Fig. 2. Screening ES clones by Southern analysis. Genomic DNA was isolated from ES clones that had been electroporated with targeting DNA and selected as described in the text. Left panel: DNA was digested with *Bam*HI, separated on a 0.7 % agarose gel and transferred to a nitrocellulose membrane for Southern hybridization. The membrane was hybridized with the completely internal probe (a). Right panel: DNA was digested with *Sca*I, separated on a 0.5 % gel, transferred to nitrocellulose and hybridized with probe (c). (+) = untargeted wild type allele; (LUC^{floxed}) = targeted *mdr*1a/LUC^{floxed} allele.

before injecting it into blastocysts to produce chimeras for germ-line transmission. Strong chimeras were obtained and these chimeric blastocysts were injected into pseudo-pregnant females to generate the founder mice.

d. Test targeted ES cells (LUC reporter) for Cre-dependent recombination and expression of LUC - Southern analysis, Northern analysis, luciferase assay.

Fig. 3. Screening recombinant ES clones by Southern analysis. Genomic DNA was isolated from *mdr*1a/LUC^{floxed} clones that had been electroporated with CAG-Cre. DNA was digested with *Eco*RI, separated on 0.7% gels and transferred to nitrocellulose membranes. Membranes were hybridized with an internal probe (b). (+) = untargeted wild type allele; (LUC^{floxed}) = floxed LUC allele; LUC^{rec} = recombined LUC allele.

To determine if Cre recombinase promotes LUC rearrangement in our ES cells, we electroporated a CAG-Cre vector (CAG: cytomegalovirus immediate early enhancer-chicken β-actin hybrid promoter)[52] into the LUC-targeted ES clone 132. Two days after electroporation, cells were harvested, plated at 500 cells/10cm dish, and cultured for 8 days without selection. Forty-eight clones were then picked from each dish, expanded, and an aliquot of each was stored for future use or analyzed for removal of floxed sequences and appropriate placement of LUC in-frame with the Pgp translation start site. For this analysis, genomic DNA was prepared from each Cre-transfected clone, digested with *Eco*RI and screened by Southern analysis, using probe b. Figure 3 shows a representative screening, out of a total of 44 clones screened. Fifty percent of the ES clones screened had undergone Cre mediated recombination, consistent with reported efficiencies of Cre-mediated recombination.[3]

To begin to determine if LUC expression is a faithful reporter of *mdr*1a in our targeted cells, we first needed to know if *mdr*1a is normally expressed in these cells. To date, *mdr*1a expression in ES cells has
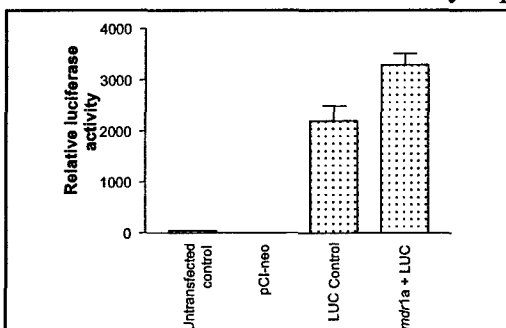
Fig. 4. Expression of *mdr*1a/LUC fusion cassette
Renilla luciferase was fused in-frame with the translated sequences of *mdr*1a exon 2 and subcloned into the mammalian expression vector pCI-neo (Promega). NIH3T3 cells were transfected with the *mdr*1a/LUC plasmid, pCI-neo containing the luciferase cDNA (positive control) or vector only (negative control). Two days after transfection the cells were harvested, lysates were prepared and bioluminescence assays performed using the Turner Design System. Results were normalized against untransfected cells. A plasmid that constitutively expressed β-gal was used to determine transfection efficiency. The *mdr*1a/LUC cassette under the control of a CMV promoter expressed luciferase at levels higher than the positive control. These data represent duplicate

not been reported. We therefore performed RT-PCR analysis on total RNA extracted from ES cells to determine if *mdr*1a expression was detectable in mouse ES cells. To prevent their differentiation and promote proliferation, ES cells are cultured with STO cells that secrete leukemia inhibitory factor. Consequently, samples of ES cells are effectively "contaminated" with STO cells that may express *mdr*1a and this had to be considered when designing the RT-PCR assay. RNA was prepared from ES + STO cells (10 % STO) and from STO cells alone; an *mdr*1a specific primer was used for the reverse transcriptase step for cDNA synthesis. To ensure that the PCR was within the linear range, cycle studies were performed on ES + STO cDNA and STO cDNA using both *mdr*1a primers and GAPDH primers as a control. Results indicated that *mdr*1a is expressed in mouse ES cells (data not shown).
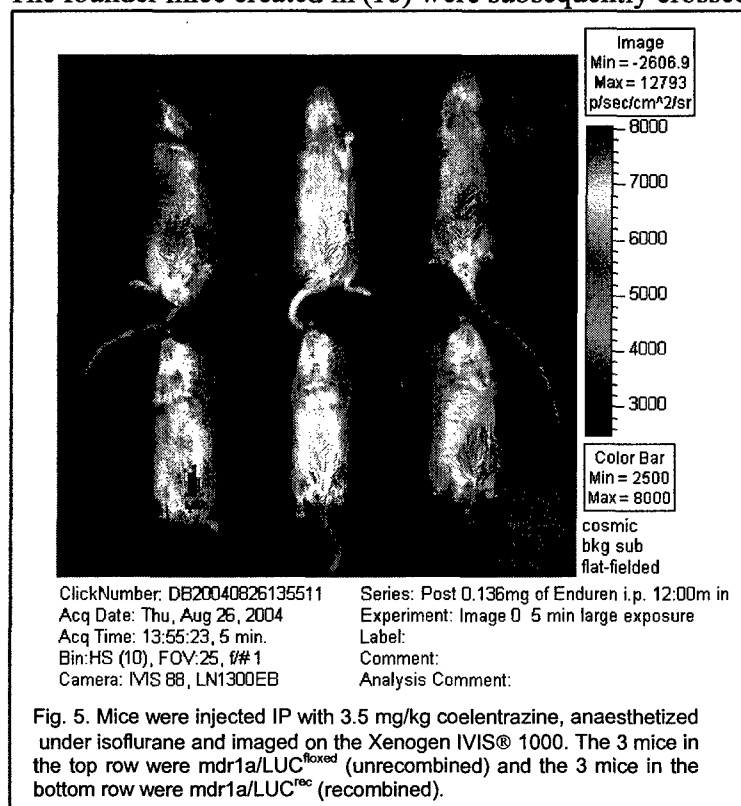
To ensure that *Renilla* luciferase was correctly transcribed and translated in the context of the *mdr*1a exon2, it was necessary to test the *mdr*1a-exon2/LUC cassette, the eventual product of Cre recombination, *in vitro* (Fig. 4). Since the targeting vector did not contain the complete *mdr*1a promoter, it was necessary to sub-clone the *mdr*1a-exon2/LUC cassette into the mammalian expression vector pCI-neo (Promega). NIH3T3 cells were transfected with the *mdr*1a-

exon2/LUC plasmid, pCI-neo containing the luciferase cDNA (positive control), or vector only (negative control). To determine transfection efficiency each group was transfected with a plasmid that constitutively expressed β-gal. Two days after transfection the cells were harvested, lysates prepared and bioluminescence assays performed using the Turner Design System (according to manufacturer's instructions). Results were corrected for β-gal activity and then normalized against untransfected cells. Background luciferase activity detected in untransfected cells and those transfected with vector only was minimal. Luciferase activity was detected from both the control pCI-neo/LUC plasmid and the *mdr*1a-exon2/LUC plasmid, with higher levels from the latter, at least in this experiment. The results are representative of duplicate experiments.

      *e.   Cross knock-in mice (1c) with CMV-Cre mice and test for recombination of reporter cassette at the DNA level; LUC expression ex vivo; LUC expression in vivo -- Southern and Northern analysis, luciferase assays, BL imaging. [50 mice, including positive and negative controls for Cre recombination] **Deliverable** = mice with ubiquitous recombined mdr1a/LUC alleles*

The founder mice created in (*1c*) were subsequently crossed with a strain which expresses Cre ubiquitously (CMV-Cre) and the offspring were analyzed for successful recombination by PCR using tail DNA. Mice positive for Cre-recombination were then analyzed for LUC expression using Xenogen's IVIS® imaging system and from 15 mice screened, a single mouse was positive for LUC signal in the abdominal region (Fig. 5). Furthermore, treating mice with drugs known to induce *mdr*1a had no effect on LUC expression. To ensure that lack of signal was not due to a sensitivity issue, LUC expression was examined *ex vivo* in tissues where *mdr*1a expression is normally observed. Although LUC expression was detected in the stomach of one additional mouse, it appears unlikely that *in vivo* expression of LUC will be detectable in our current mouse strain due to sensitivity limitations. To confirm this hypothesis, RT-PCR will be used to analyze *mdr*1a and LUC expression in dissected organs. The positive LUC signal in one of our mice may be associated with an inflammatory response; this is being investigated in light of potential implications for induced *mdr*1a expression under physiological stress conditions.

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Analysis Comment:

Fig. 5. Mice were injected IP with 3.5 mg/kg coelentrazine, anaesthetized under isoflurane and imaged on the Xenogen IVIS® 1000. The 3 mice in the top row were mdr1a/LUC^floxed (unrecombined) and the 3 mice in the bottom row were mdr1a/LUC^rec (recombined).

To optimize LUC expression, we have now redesigned the targeting vectors to contain either the humanized *Renilla* or firefly luciferase reporter genes. In addition, the Cre lox system has been utilized to remove the neo selection marker prior to generating transgenic mice, and synthetic polyA signals have been incorporated into the new vectors. It is hoped that these targeting vectors will result in detectable basal levels of luciferase expression in knock-in mice.

      f. Cross knock-in mice (1c) with tissue-specific Cre donators and test for tissue-specific

recombination and expression as above. [50 mice per cross] **Deliverable** *= mice with tissue-specific mdr1a/LUC alleles.*

Because it is unlikely that the tissue-specific *mdr1a/LUC* mice will have detectable *in vivo* LUC signal given that we were unable to detect LUC in the mice generated in 1e, Task 1f will be performed once the re-engineered vector has been successfully knocked-in and validated in the ubiquitously expressing Cre mice.

Task 2. Determine if mdr1a/LUC is drug-inducible in normal tissues (months 10-18)

a.   Inject drugs into ubiquitous mdr1a/LUC (1e) mice and perform BL imaging over time (hours) -- 6 drugs plus vehicle control, 3 doses per drug, 6 mice per treatment group. [126 mice]

As described in Task 1e above, *in vivo* LUC signal was undetectable in all but one mouse under both basal and drug-treated conditions. In particular, we tested a known and potent inducer of *mdr*1 expression in mouse and human cells, pregnenolone-16α-carbonitrile, and saw no change LUC signal *in vivo*. While we did not observe inducible luciferase expression, we believe this was due, in part, to inadequacies of the targeting vector design. We will resume these experiments once the re-engineered vector has been successfully knocked in and validated.

b.   Inject drugs into tissue-specific mdr1a/LUC mice (from 1f) and perform BL imaging over time (hours) -- 6 drugs plus vehicle control, 2 doses per drug (based on 2a), 6 mice per treatment group. [84 mice]

We were unable to perform these proposed experiments proposed since we did not create the tissue-specific *mdr*1a/LUC mice (see Task 1f). We will do these in the future once the re-engineered vector has been successfully knocked in and validated.

c.   Cross mdr1a/LUC mice with PXR(-/-) knockout mice and inject with drugs as above. Perform BL imaging over time (hours) -- 3 drugs plus vehicle control, 2 doses per drug, 6 mice per treatment group. Only if positive results in 2a and 2b. [48 mice]

We also did not perform the cross with PXR(-/-) mice, for reasons stated in 2b.

Task 3. Establish the model to determine if mdr1a/LUC expression is turned on during breast cancer progression and/or treatment (months 1-18). Initial experiments only for Aim 3.

a.   Establish conditions for breast tumor formation in a variety of genetic backgrounds (conditional knockout of tumor suppressors, crossed with mammary-specific Cre donators). Monitor for tumor formation over time (months).
b.   Cross LUC knock-in mice with mammary-specific Cre donators and test for tissue-specific recombination (mdr1a/LUC) as above. [50 mice] **Deliverable** *= mice with mammary-specific mdr1a/LUC allele and tissue-specific Cre maintained*
c.   Time permitting, cross mice from 3b with conditional tumor suppressor knockout mice, monitor for tumors and mdr1a/LUC expression over time -- caliper measurements, BL imaging. [15 mice/cross] **Deliverable** *= mice with mammary-specific mdr1a/LUC allele and mammary-specific tumor suppressor knockout.*

As described in Task 1e above, *in vivo* LUC signal was undetectable in all but one mouse under both basal and drug-treated conditions. Therefore, we were unable to perform the experiments proposed in Task 3. Task 3 will be addressed in the future once the re-engineered vector has been successfully knocked in and validated.

Key Research Accomplishments
- We engineered a targeting vector to allow insertion of a reporter (luciferase or HSV-tk) into the genomic locus of the mouse *mdr*1a gene.

- We created mouse embryonic stem cells in which a gene replacement/knock-in strategy was used to insert luciferase into the mouse *mdr*1a genomic locus.

- We demonstrated that luciferase expression in these cells requires Cre recombinase to bring luciferase in-frame with the translational start site of the *mdr*1a gene product.

- We have shown that the recombined configuration of *mdr*1/LUC, in its cDNA form, encodes a functional LUC protein with luciferase activity.

- We created both founder and Cre-recombinase expressing mouse strains for use in the *in vivo* imaging experiments.

Reportable Outcomes
1) Abstracts
- Brown D.A., Kane, S.E., Synold, T.W. Genetic controlled reporter gene expression. 3rd Ann. Meeting of Soc. Mol. Imaging, Mol. Imaging. Volume 3(3), 2004.

- Donna A. Brown, Timothy. W. Synold, and Susan E. Kane. Genetic locus-controlled reporter gene expression. Ann. Meeting of Am. Assoc. Cancer. Res. Submitted, Nov. 2004.

2) Presentations
- Brown D.A., Kane, S.E., Synold, T.W. Genetic controlled reporter gene expression. 3rd Annual Meeting of The Society for Molecular Imaging, St. Loius , MO, Sept. 2004.

3) Mouse Models
- *mdr*1a/LUC^{floxed} (unrecombined)
- *mdr*1a/LUC^{rec} (recombined)

4) Funding Applied For
- RFA-05-002 Innovative Technologies for Molecular Analysis of Cancer (R21). Grant title – "Genetic locus-controlled mdr1a reporter gene expression". Submitted, Feb. 2004.

Bibliography
Abstracts
- Brown D.A., Kane, S.E., Synold, T.W. Genetic controlled reporter gene expression. 3rd Ann. Meeting of Soc. Mol. Imaging, Mol. Imaging. Volume 3(3), 2004.

- Brown D.A., Synold T.W., Kane S.E.. Genetic locus-controlled reporter gene expression. Ann. Meeting of Am. Assoc. Cancer. Res. Submitted, Nov. 2004.

List of Personnel
- Timothy W. Synold, Pharm.D. – Principal Investigator
- Donna A. Brown, Ph.D. – Postdoctoral Fellow
- Jenny Glavin – Research Technician

Conclusions

The human *MDR*1 gene has long been implicated as one mechanism of drug resistance in some cancers[4-6], but it has been difficult to prove the exact link between *MDR*1/Pgp and the clinical manifestations of drug resistance. There are several reasons for this: 1) Rigorous testing of *MDR*1 in breast cancer requires repeated, longitudinal biopsies that are difficult to obtain. 2) Even if biopsy material were available, tumor tissue is often contaminated with normal breast tissue and adequate biological material is generally not achievable. 3) Breast cancer treatment protocols under which *MDR*1 is studied usually include drugs that are substrates for Pgp (e.g., taxanes, *Vinca* alkaloids, anthracyclines,) and those that are not (e.g., platinated compounds, methotrexate), making it difficult to conclude any meaningful relationships between *MDR*1 expression and tumor response or survival. 4) Studies with *MDR*1/Pgp modulators, which attempt to inhibit tumor Pgp function and thereby improve drug efficacy, also affect Pgp function in normal tissue in a way that impacts pharmacodynamics and pharmacokinetics. This makes it difficult to evaluate the contribution of tumor Pgp vs. normal-tissue Pgp to therapeutic outcome. 5) A good animal model has not been available to study these issues of *MDR*1/Pgp expression and function.

Our results to date (summarized above) have established the feasibility of inserting a reporter gene (LUC) into the genomic locus of the mouse *mdr*1a gene, thus allowing us to generate a mouse model that should express LUC in tissues that normally express mdr1a. Furthermore, we have demonstrated the feasibility of utilizing a targeting vector that requires the presence of Cre-recombinase to bring the reporter in frame with the translation start site, and in its cDNA form, encodes a functional LUC protein. The benefit of this approach is that we should ultimately be able to generate tissue-specific LUC-expressing mice to allow for tissue-specific gene regulation studies. Unfortunately, despite successful targeting and recombination, we were unable to detect a LUC signal in our mice either under basal or drug-treated conditions. Interestingly, however, the positive LUC signal seen in one of our mice may have been associated with an inflammatory response; this is being investigated in light of potential implications for induced *mdr*1a expression under physiological stress conditions.

Our current hypothesis is that, although LUC expression was detected in a single live mouse and in the stomach of one additional mouse ex vivo, our current model either is not sufficiently sensitive to be useful for future imaging studies or is deficient in one or more vector design element such that recombination and/or mRNA expression is too inefficient. To optimize sensitivity and LUC expression, we are currently redesigning the targeting vector in several ways: 1) the vector will contain either the humanized *Renilla* or firefly luciferase reporter genes to determine which gives us the greatest sensitivity in our system; 2) the neo selection marker will be re-engineered such that the Cre-lox system will remove the marker prior to generating transgenic mice; and 3) synthetic polyA signals are being incorporated to ensure more efficient transcription termination immediately downstream of the LUC open reading frame. It is hoped that this revised targeting vector will result in detectable basal levels of LUC expression in the appropriate tissues of knock-in mice and, in turn, regulated expression under physiological conditions of interest.

"So What"

We have proposed to use *mdr*1a as a model for studying *mdr*1 gene regulation, using a bioimaging reporter that has been targeted to the chromosomal locus of the *mdr*1a gene. With this model, we hope to be able to address many of the unanswered questions about *mdr*1 gene regulation and its role in breast cancer. Using our approach, we will ultimately be able to study *mdr*1 in individual tissues – those that normally express the gene (e.g. liver and intestine) and those that do not (e.g. mammary tissue) – under conditions of drug delivery, imposition of other stresses, during tumorigenesis, and in a variety of genetic backgrounds (through the propitious mating of *mdr*1a/reporter knock-ins with appropriate gene knock-out mice) that will reveal the

10

necessity for certain genes/proteins for a given physiologic response. In a longer-term view, it might even be possible to explore the influence of specific cis-acting elements on gene regulation (tissue specificity of basal and/or inducible expression) by targeting mutant promoter elements into the *mdr*1a/LUC locus via a second homologous recombination event.

By targeting a reporter directly to the *mdr*1a genomic locus (rather than randomly integrating it), by studying regulation of the mouse *mdr*1a gene in the context of the mouse genetic background, and by introducing the Cre-dependent element of spatial and temporal control of the knock-in event, we hope to develop a more biologically and physiologically appropriate system for studying the regulation of *mdr*1a gene expression. The availability of the human *MDR*1/LUC model will be an important comparator for future applications of the *mdr*1a/LUC mice developed under this proposal. Importantly, the *mdr*1a/reporter model will serve as a proof-of-principle which, if successful, can be applied to a multitude of other biologically and clinically relevant problems. These can include questions related to *mdr*1a (e.g., testing the effect of chemoprevention on *mdr*1a induction), but will also encompass applications to virtually any other gene of interest and conditions that impact transcriptional regulation of that gene, be they environmental signals, hormonal signals, growth factor signals, angiogenesis signals, survival or death signals, just to name a few of the possibilities.

This work represents a novel approach to the question of gene regulation, using a biologically significant gene (*mdr*1a) as a model. If successful, this animal model will provide new, heretofore, unattainable information about the role of *mdr*1a in drug resistance and tumorigenesis. It would also be the first demonstration of monitoring regulated expression of an endogenous chromosomal locus *in vivo*, in real time, and under manipulated physiological conditions.

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Appendices

1) Brown D.A., Kane, S.E., Synold, T.W. Genetic controlled reporter gene expression. 3rd Ann. Meeting of Soc. Mol. Imaging, Mol. Imaging. Volume 3(3), 2004.

Genetic locus-controlled reporter gene expression. Donna A. Brown[1*], Susan E. Kane[2] and Timothy. W. Synold[1].
[1]City of Hope Comprehensive Cancer Center, Department of Medical Oncology, 1500 E. Duarte Rd. Duarte, CA 91010. [2]California State University, Los Angeles, Department of Biology, 5151 State University Drive, Los Angeles, CA 90032-8253.

The relationship between the over-expression of the multidrug resistance-1 (*MDR*1) gene and clinical drug resistance in human cancers remains tentative. Good models to study the links between drug resistance and *MDR*1 expression have not been available. Using small animal imaging tools we now have designed a mouse model that will allow us to examine *mdr*1a (a homologue of human *MDR*1) expression *in vivo*, in real time, and in response to developmental, physiological, and environmental signals. Through homologous recombination we have inserted the *Renilla* luciferase (LUC) gene into the genomic locus placing it under the control of the *mdr*1a promoter. This model has been designed to allow us to examine tissue-specific expression of *mdr*1a through use of Cre-loxP technologies. To our knowledge our model is unique and therefore, if successful, will be the first system of its kind to examine locus controlled, regulated reporter gene expression in specific tissues *in vivo*.

The following milestones have been accomplished: 1) we have engineered a targeting vector to insert LUC into the genomic locus of the mouse *mdr*1a gene in a way that makes in-frame expression of the reporter conditional on Cre-mediated recombination. 2) we have created mouse embryonic stem (ES) cells and demonstrated homologous recombination into the mouse *mdr*1a genomic locus. 3) we have shown that Cre transfection into these cells mediates LUC recombination into the first coding exon of *mdr*1a. 4) *mdr*1a/LUC ES cells have been used to generate the corresponding knock-in mice. 5) *mdr*1a/LUC mice have been crossed with ubiquitous Cre-donator mice and resulting off-spring have been screened for Cre-recombination; positives have been obtained, and analysis of these mice is in progress.

2) Brown D.A., Synold T.W., Kane S.E. Genetic locus-controlled reporter gene expression. Ann. Meeting of Am. Assoc. Cancer. Res. Submitted, Nov. 2004.

Genetic locus-controlled reporter gene expression. Brown D.A., Synold T.W., and Kane S.E.
City of Hope Comprehensive Cancer Center, Department of Molecular Medicine, 1500 E. Duarte Rd. Duarte, CA 91010.

Despite advances in the understanding of transcriptional regulation of *MDR*1 expression *in vitro*, in the context of the whole body, the regulatory mechanisms of *MDR*1 expression are poorly understood. Our lack of understanding is due to our inability to measure changes in gene expression under real physiological conditions and in real time, and until now good models have not been available. Using small animal imaging tools we have designed a mouse model that will allow us to examine *mdr*1a (a homologue of human *MDR*1) expression *in vivo*, in real time, and in response to developmental, physiological, and environmental signals, with a view to resolving unanswered questions pertaining to the over-expression of *MDR*1 and clinical drug resistance.

Through homologous recombination we have inserted the *Renilla* luciferase (LUC) gene into the *mdr*1a genomic locus and thus under the control of the *mdr*1a promoter. Cre-loxP technology was incorporated

into the model such that in-frame expression of the LUC reporter is conditional on Cre-mediated recombination and *mdr*1a expression. By controlling Cre expression we can examine tissue-specific expression of *mdr*1a. To our knowledge our model is unique and therefore, if successful, will be the first system of its kind to examine locus controlled, regulated reporter gene expression in specific tissues *in vivo*.

We have created mouse embryonic stem (ES) cells and demonstrated homologous recombination of our targeting vector into the mouse *mdr*1a genomic locus; the ES were used to generate knock-in mice that have been crossed with ubiquitous Cre-donator mice. Mice positive for Cre-recombination have been analyzed for LUC expression using Xenogen's IVIS® imaging system and from 14 mice screened, one mouse was positive for LUC signal in the abdominal region. Treating mice with drugs known to induce *mdr*1a had no effect on LUC expression. To ensure that lack of signal was not due to a sensitivity issue, LUC expression was examined *ex vivo* in tissues where *mdr*1a expression is normally observed. Although LUC expression was detected in the stomach of one mouse, it appears unlikely that *in vivo* expression of LUC was undetectable due to sensitivity limitations. To confirm this hypothesis RT-PCR will be used to analyze *mdr*1a and LUC expression in dissected organs. The positive LUC signal in one of our mice may be associated with an inflammatory response; this is being investigated in light of potential implications for induced *mdr*1a expression under physiological stress conditions.

To optimize LUC expression, we have redesigned the targeting vectors; vectors now contain both the humanized *Renilla* or firefly luciferase reporter genes. The Cre lox system has been utilized to remove the neo selection marker prior to generating transgenic mice. Synthetic polyA signals have also been incorporated into the new vectors. It is hoped that these targeting vectors will result in detectable basal levels of luciferase expression in knock-in mice.